JOURNAL OF BONE AND MINERAL RESEARCH
Volume 12, Number 12, 1997
Blackwell Science, Inc.

Functional Characterization of the Human Biglycan 5′-Flanking DNA and Binding of the Transcription Factor c-Krox

ANNE-MARIE HEEGAARD,[1] PAMELA GEHRON ROBEY,[2] WALTHER VOGEL,[3] WALTER JUST,[3] RUSSELL L. WIDOM,[4] JØRGEN SCHØLLER,[1] LARRY W. FISHER,[2] and MARIAN F. YOUNG[2]

ABSTRACT

The transcriptional regulation of human biglycan expression under normal and pathological conditions was studied. The 5′-flanking regions of the human and mouse genes were isolated and analyzed; the two promoter regions share 81% identity. Both promoters are without a TATA and CAT box and contain multiple Sp1 sites. Human dermal fibroblasts were transiently transfected with progressive deletional human biglycan 5′-flanking DNA-CAT constructs, and a significant variation in activity among the individual constructs was found. A small deletion in several cases caused a more than 2-fold increase or decrease in promoter activity, thereby mapping the target sites for repressors or activators. Human biglycan expression is reduced in females with Ullrich-Turner syndrome (45,X) and increased in individuals with supernumerary sex chromosomes, and it has been speculated that biglycan plays a role in the short stature phenotype of Turner syndrome. Analysis of the transcriptional regulation of biglycan in individuals with sex chromosome anomalies showed that a –262 to –218 region of the biglycan promoter was differentially regulated. This region was extensively analyzed by DNAse footprinting and electrophoretic mobility shift assays, and a putative binding site for the transcription factor c-Krox was discovered. The binding of c-Krox to a site located at approximately –248 to –230 in the human biglycan promoter was confirmed by using extracts from COS cells expressing recombinant human c-Krox. The expression of c-Krox in bone was then examined by reverse-transcribed polymerase chain reaction and Northern blotting analysis; an ~3.4 kb transcript was detected in primary osteoblastic cells, in MG-63 cells, and in human bone marrow stromal cells. This is the first detection of c-Krox in bone cells, and it suggests that c-Krox, like another member of the Krox family, Krox-20, might play a regulatory role in bone. (J Bone Miner Res 1997;12:2050-2060)

INTRODUCTION

BIGLYCAN is a small, leucine-rich, proteoglycan that, in the developing human fetus, is present in bone, skin, and a few other specialized connective tissues.[1] The function of biglycan is unknown, and comparison of the amino acid sequence of human, mouse, rat, and bovine biglycan core proteins shows it to be highly conserved among species (more than 90% identity), suggesting an essential biological function.[2] Biglycan has been shown to bind to transforming growth factor-β1, -2, and -3 (TGF-β)[3] as well as type I collagen fibrils.[4] It has been proposed to participate in the structural organization of the extracellular matrix and to be involved in the regulation of TGF-β activity.[5] In addition, the localization of biglycan in developing bone to growth plates and preosteogenic cells has pointed toward a role in osteoblast differentiation.

The human biglycan gene has been isolated and character-

[1]Center for Clinical and Basic Research, Ballerup, Denmark.
[2]Bone Research Branch, NIDR, National Institutes of Health, Bethesda, Maryland, U.S.A.
[3]Abteilung Medizinische Genetic der Universität Ulm, Ulm, Germany.
[4]Boston University School of Medicine, Arthritis Center, Boston, Massachusetts, U.S.A.

ized[6,7]; it contains eight exons and has been mapped to the long arm of the X chromosome to a localization within the Xq28 region.[8] Recently, it was shown that biglycan mRNA and protein expression is affected in individuals with sex chromosome anomalies.[9] In fibroblasts from females with Ullrich-Turner syndrome (45,X) there was a significant decrease in biglycan expression to 55% of the level observed for cells from normal male (46,XY) and female (46,XX) donors and an increased expression in cells from individuals with additional sex chromosomes (e.g., 47,XXY or 49,XXXXY).[9] This suggested that biglycan mimics a pseudoautosomal gene that should be present both on the X and Y chromosomes and not subject to X inactivation. Because this is not the case, a hypothesis was proposed that suggested the presence of a biglycan regulatory factor in one of the pseudoautosomal regions on the sex chromosomes. The pseudoautosomal region (PAR1) on the telomere of the X and Y chromosomes[10] has been shown to contain gene(s) that are involved in the Ullrich-Turner phenotype[11,12] and part of the determinant of stature.[10] It has therefore been speculated that since biglycan potentially has a role in skeletal development it might be one of the molecules linking a regulatory "Turner gene" to growth.[9]

To elucidate the mechanisms involved in the transcriptional regulation of biglycan, we cloned the 5′ regulatory regions of the human and mouse biglycan genes and found that the two promoters are 81% homologous. Progressive deletions of the human biglycan 5′ region were subcloned into a chloramphenicol acetyltransferase (CAT) expression vector and transfected into human dermal fibroblasts derived either from normal males or females or from individuals with Ullrich-Turner syndrome or with supernumerary sex chromosomes. Target areas for either repressors or activators of biglycan transcription were identified. A biglycan promoter construct, which contained an AP2 site and a putative binding site for the transcription factor c-Krox, showed increased transcriptional activity when transfected into fibroblasts with the genotype 47,XXX or 49,XXXXY. Subsequently, evidence was provided that c-Krox, but not AP2, binds to this region in the biglycan promoter. c-Krox was recently discovered to be a zinc finger transcription factor that can increase the transcriptional activity of the mouse α1 (I) collagen promoter through its binding to two GC-rich sequences.[13] In the mouse, c-Krox mRNA is predominantly expressed in skin and it colocalizes with α1 (I) collagen mRNA during mouse development.[14] Even though α1 (I) collagen is highly expressed both in fibroblasts and osteoblasts, no expression of c-Krox was observed in mouse bone, and it was suggested that c-Krox specifically is involved in the transcriptional regulation of collagen in fibroblasts. In this paper, we present evidence that c-Krox is also involved in the transcriptional regulation of human biglycan and that c-Krox mRNA is expressed in human osteoblastic cells and bone marrow stromal cells.

MATERIALS AND METHODS

Cloning of human and mouse biglycan 5′-flanking DNA

Five hundred thousand plaques from a human Lambda FIX II genomic library (Stratagene, La Jolla, CA, U.S.A.) were screened with the human biglycan cDNA, clone P16,[15] as previously described.[6] Ten positive clones were isolated and analyzed in Southern blots with a biglycan promoter-specific oligonucleotide (−499 to −481) as probe. A positive 1.9 kbp *Sst*I fragment was then isolated and subcloned into both Bluescript II SK$^-$ and M13 mp19 (Stratagene) and then DNA sequenced in both directions. When necessary, deoxyinosine 5′ triphosphonate (dITP) was used instead of deoxyguanosine triphosphonate (dGTP) to resolve the sequence of regions with strong compressions (Protocol for Sequence®, Version 2.0 DNA sequencing kit, United States Biochemical, Cleveland, OH, U.S.A.). Mouse biglycan genomic DNA was obtained from a 129/SVJ mouse genomic library (kindly donated by Dr. T.C. Doetschman). The rat biglycan cDNA (a kind gift from Dr. K.L. Dreher)[16] was used to screen 500,000 plaques. Five clones were isolated and analyzed by Southern blotting, and a 1.0 kbp *Bam*HI–*Sac*I fragment was isolated, subcloned, and both strands DNA sequenced.

Construction of biglycan promoter-CAT vectors

Ten micrograms of pUC18 vector (pOBCAT7/pOBCAT5; a generous gift from Dr. C.C. Baker),[17] containing the SV40 early promoter, the SV40 late splices, and the CAT gene, was cleaved with *Bgl*II and *BamH*I (pOBCAT7) or *Hind*III and *Kpn*I (pOBCAT5), thereby releasing the SV40 early promoter and the SV40-late-16S/19S splice donor and the SV40-late-19S splice acceptor, but leaving the SV40-late-16S splice acceptor. The DNA was then dephosphorylated with bacterial alkaline phosphatase to prevent religation. Eleven deletional fragments of the biglycan promoter with the appropriate restriction sites were generated using the polymerase chain reaction (PCR). The 5′ end primers created either a *Bgl*II (my157, my160, my162, my161) or a *Hind*III (my281, my282, my283, my284, my285, my286) restriction site and in addition corresponded to the following sites in the biglycan promoter: my281, –1208 to –1186; my282, −873 to −856; my283, −801 to −779; my284, –713 to −692; my285, −675 to −657; my286, −660 to −637; my157, −497 to −476; my160, −428 to −407; my162, – 262 to –241; my161, –218 to –198. Three different 3′ end primers were used; my158 corresponded to the reverse and compliment of biglycan intron 1 from +204 to +224 and created a *BamH*I restriction site, my287 contained almost the same sequence as my158 but created a *Kpn*I site, and My159 corresponded to the reverse and compliment sequence of exon 1 from +65 to +85 and created a *BamH*I restriction site. The primers were purchased either from Synthecell (Rockville, MD, U.S.A.) or Midland Certified Reagent Company (Midland, TX, U.S.A.). A 1.9 kbp *Sst*I fragment of biglycan genomic DNA (–1218 to +642) previously subcloned into Bluescript II SK- (Stratagene) was used as template for the reactions. Eleven PCR reactions were performed using standard procedures recommended by the manufacturer (GeneAmp DNA Amplification Reagent Kit, Perkin Elmer Cetus, Norwalk, CT, U.S.A.) with the following primer combination: my281/my287, my282/my287, my283/my287, my284/my287, my285/my287, my286/my287, my157/my158, my157/my159, my160/my158, my162/my158,

and my161/my158. The PCR products were treated with Klenow enzyme, cleaved with either *Bgl*II and *Bam*HI or *Hind*III and *Kpn*I, and isolated by electrophoresis in 1.5% TBE agarose gels followed by NA-45 paper extraction. The treated PCR products were then ligated to the linearized CAT vector and the inserts sequenced to verify that no mutations had been introduced. All constructs were cesium chloride gradient purified twice, precipitated multiple times, and resuspended in deionized H_2O.

Cells

Human fibroblasts were obtained from skin biopsies from patients and healthy controls seen at the outpatient clinic of the Department of Medical Genetics at the University of Ulm, Germany as previously described.[9] Routine karyotyping was performed on all cell lines. The cells were used at passages 8–16. The WI-38 fibroblast cell line was a generous gift from Dr. Ulla Wewer. The osteoblastic cells were derived from collagenase-treated human trabecular bone fragments using surgical waste according to National Institutes of Health-Institutional Review Board approved procedures and cultured in low calcium medium as described previously.[18] Human bone marrow stromal cells were obtained and cultured as described previously.[19]

Transfection experiments

The DNA was introduced into the cells using a calcium phosphate method described previously.[20] Briefly, 15 μg of biglycan promoter-CAT-vector DNA and 2.3 μg of CMV-promoter-luciferase vector DNA[21] in a total volume of 450 μl of H_2O was mixed with 50 μl of 2.5 M $CaCl_2$ and then slowly added to 500 μl of 2× HEPES buffer. After 20 minutes of incubation at room temperature, the DNA solution was added to the fibroblasts (100-mm dishes, 13,000 cells/cm^2, 9 ml of complete medium with 10% heat inactivated fetal calf serum from Hyclone Laboratories, Logan, UT, U.S.A.) and incubated at 37°C and with 5% CO_2. After ~14 h, the cells were washed twice in phosphate buffered saline, and fresh complete medium was added. The cells were harvested 48 h after the addition of DNA. A CAT assay System (Promega E1000, Promega Biotech, Co., Madison, WI, U.S.A.) was used for measuring CAT activity. The cell extracts and the CAT enzyme assays were performed as described in the Promega protocol using freeze-thaw cycles, the standard reaction enzyme assay, and liquid scintillation counting. For each standard reaction, 50 μl of cell extract, 5 μl of ^{14}C-chloramphenicol, 5 μl of *n*-butyryl Coenzyme A, and 65 μl of 0.25 M Tris-HCl, pH 8.0, were used. The samples were incubated for 16–18 h. The luciferase activity was measured using a luminometer (Monolight 2010, Analytical Luminescence Laboratory, San Diego, CA, U.S.A.). Ten microliters of cell extract was added to 350 μl of assay buffer (25 mM glycylglycine, pH 7.8, 25 mM $MgSO_4$, 2 mM ATP, 10% glycerol, and fresh 1 mM DTT), and the instrument injected 100 μl of 1 mM D-luciferin-potassium salt (Analytical Luminescence Laboratory). Two samples were measured from each cell extract.

RNAse protection assay

Total RNA was isolated (as described below) from MG-63 cells which had been transfected with 15 μg of biglycan-promoter-CAT vector per dish. The RNA was dissolved in TE buffer and treated with RQ1 DNAse (Promega) in the presence of 10 mM $MgCl_2$, 1 mM DTT, and 5 U of RNAsin (Promega) as previously described.[22] Poly(A) RNA was prepared using a Poly(A) Quik mRNA isolation kit (Stratagene) according to the manufacturer's instructions. To investigate the exact sequence of the biglycan-CAT vector transcripts, total RNA from MG-63 cells transfected with the –262 biglycan-promoter-CAT-construct was reverse transcribed and subsequently used in a PCR. A 226 bp product was generated corresponding to the expected size if the biglycan-CAT transcripts had undergone splicing. The up- and downstream primers corresponded to +21 to +40 (5′-GAGTGAGTAGCTTTCGG-3′) in the biglycan exon 1 and to the N-terminal of the CAT gene (5′-CTCCATTTTAGCTTCTTAGC-3′), respectively, and the reverse transcribed polymerase chain reaction (RT-PCR) was performed as recommended by the manufacturer (Perkin Elmer, RNA PCR kit). After TA-cloning (Invitrogen, San Diego, CA, U.S.A.), the RT-PCR product was DNA sequenced. The sequence analysis showed that the biglycan intron and the SV40 DNA had been removed by splicing at the biglycan splice donor site and the SV40 splice acceptor site. To generate an antisense probe for the RNAse protection assay, an *Acc*I–*Spe*I fragment of the biglycan gene (–185 to +98) was inserted into the *Acc*I–*Spe*I sites in pBluescript KS (Stratagene), and a *Spe*I–*Xba*I fragment of the RT-PCR product from the transfected cells was then inserted into the *Spe*I–*Xba*I sites of the same vector, thus generating an *Acc*I–*Xba*I fragment containing 185 bp upstream biglycan DNA, biglycan exon 1, and 67 bp of the CAT gene. The vector was linearized with *Xho*I, gel purified, and 0.3 μg was in vitro transcribed using T7 RNA polymerase and the Riboprobe In Vitro Transcription System (Promega). The RNAse protection assay was performed using the RPA II Ribonuclease Protection Assay Kit (Ambion, Inc., Palo, Alto, CA, U.S.A.). The products were resolved by 6% denaturing polyacrylamide gel electrophoresis and visualized by autoradiography.

Nuclear and whole cell extracts

A modification of the method of Dignam et al.[23] was used to prepare nuclear extracts from confluent cultures of human skin fibroblasts. The following changes in the method were made: One Complete protease inhibitor cocktail tablet (Boehringer Mannheim, Mannheim, Germany) per 50 ml buffer was used in all the buffers instead of polymethylsulfonyl fluoride (PMSF) and dithiothreitol (DTT). Additionally, dialysis was omitted, but the extracts were concentrated using Microcon-30-microconcentrators from Amicon according to the manufacturer's instructions. The protein concentration in the extracts was determined using a Bio-Rad Protein Assay Kit I (BioRad, Melville, NY, U.S.A.). A confluent cell culture in a 150-mm dish resulted in 35 μl of nuclear extract with a protein concentration of

~10 μg/μl. HeLa nuclear extract was obtained from Promega. Whole cell extracts from COS cells transfected with either c-Krox sense or antisense expression vectors were constructed as described elsewhere.[24]

DNAse footprinting

A −428 biglycan-promoter-CAT construct was treated with *Bgl*II and *Acc*I, thereby releasing a 240 bp biglycan promoter fragment (−428 to −188). The fragment was isolated by NA-45 paper extraction and subcloned into a Bluescript II SK⁻ vector which had been cut with *Bam*HI and *Acc*I. A large-scale preparation was made of the new construct, and the promoter fragment was then released with *Not*I and *Apa*I, which flank the *BamH*I–*Acc*I insertion sites. The fragment was purified and both ends were labeled using T4 polynucleotide kinase in a reaction that contained 8 U of enzyme, 0.3 μg of DNA, 50 μCi of [γ-^{32}P]ATP (at 3000 Ci/mmol, 10 mCi/ml), and kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine, 0.1 mM EDTA). The total volume of the reaction (20 μl) was incubated at 37°C for 30 minutes. Then an additional 8 U of the enzyme was added, and the incubation was continued for 30 minutes. To remove the 5′ end label, the DNA was digested with *Spe*I, extracted once with phenol:chloroform:isoamyl alcohol and once with chloroform: isoamyl alcohol, purified on a G-50 spin column, ethanol precipitated, and the pellet washed twice with 1 ml of 70% ethanol, dried, and resuspended in 100 μl of TE buffer. A Stratagene HotFoot buffer kit was subsequently used for the binding reactions and the DNAse treatment, which were performed essentially as recommended by the manufacturer with the following exceptions: for each reaction, the activity of the probe was 50,000–100,000 cpm, 2 μg of poly(dI–dC) was used, and the DNAse I from the kit was substituted with DNAse I from Worthington Biochemical Corporation (Freehold, NJ, U.S.A.). To identify the areas corresponding to the footprints, the probe was DNA sequenced with the New England Nuclear Maxam–Gilbert DNA sequencing system (New England Nuclear, Danvers, MA, U.S.A.). The products from the footprinting and the sequencing reactions were resolved on a denaturing 10% polyacrylamide gel and visualized by autoradiography.

Electrophoretic mobility shift assay

Double-stranded, polyacrylamide gel purified oligonucleotide probes (custom-made, DNA technology, Aarhus, or Pharmacia Biotech, Allerod, Denmark) were end-labeled with [γ-^{32}P]ATP, and then for each electrophoretic mobility shift assay (EMSA) reaction (total volume 15–17 μl), 50,000 cpm was mixed with 2 μg of poly(dI–dC) (Pharmacia), 10 μl of buffer (20 mM HEPES, pH 7.9, 20% glycerol, 50 mM KCl, 0.5 mM DTT), H_2O, and ~40 μg of nuclear protein. After 25 minutes of incubation at 30°C, 1 μl of 0.25% xylene cyanol-bromophenol blue was added to each sample, which was then resolved on a 6% nondenaturing polyacrylamide gel (6% polyacrylamide, gelshift running buffer, 2.8% glycerol, 0.1% ammonium persulfate and N,N,N′,N′-tetramethylethylene-diamine [TEMED]) in gelshift running buffer (50 mM Tris, 380 mM glycine, 2 mM EDTA, pH 8.5). The gel was dried and the bands were visualized by autoradiography.

RT-PCR and Northern blots

Total RNA was isolated using the guanidine isothiocyanate method and cesium chloride purification as previously described.[25] First strand cDNA synthesis was performed as recommended by the manufacturer (Pharmacia, First-Strand cDNA Synthesis Kit). The *Not*I-d$(T)_{18}$ primer from the kit, and 1 μg of total RNA was used for each reaction in a 15 μl reaction volume. One microliter of the single-stranded complimentary DNA product was then amplified by PCR in a 50 μl reaction volume with the following conditions: 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.001% gelatin, 0.2 mM of each dNTP, 0.4 mM forward primer (5′-CAAGACAAGCTGGTGCGCAAACG-3′), 0.4 mM reverse primer (5′-CCTCACAGGTGGCACTCA TAG-3′), 0.5 μl AmpliTaq Gold polymerase (Perkin Elmer), 1 cycle at 94°C for 11 minutes, then 26 cycles of 94°C for 1 minute, 63°C for 1 minute and 72°C for 1 minute, then 1 cycle at 72°C for 7 minutes. The PCR products were resolved by agarose gel electrophoresis (1% agarose gel) and visualized by ethidium bromide staining.

For Northern blot analysis, 20 μg of total RNA was loaded in each lane of a denaturing formaldehyde agarose gel as previously described[25] and separated by electrophoresis at 13 V for 20 h. The RNA was transferred to nitrocellulose (BA-S 85, Schleicher & Schuell, Inc., Keene, NH, U.S.A.) and cross-linked in a UV Stratalinker 2400 (Stratagene). The blot was prehybridized at 42°C for 3 h in 5× SSC, 5× Denhardt's solution, 20 μg/ml salmon sperm DNA, 50% formamide and 10% dextran sulfate, and then hybridized for 18 h at 42°C in the same solution with a [α-^{32}P]dCTP-labeled (Rediprime DNA labeling system, Amersham International, Little Chalfont, Buckinghamshire, U.K.) 1.8 kbp human c-Krox cDNA.[24] The blot was washed repeatedly in 2× SSC, 0.1% SDS, and 0.1× SSC, 0.1% SDS at room temperature and at 65°C, and after 1 week exposure visualized by a PhosphorImager SF (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

RESULTS

Cloning of human and mouse biglycan 5′ DNA

A human biglycan cDNA[15] was used to screen a human Lambda FIX II genomic library, and 10 clones were isolated and analyzed. The clones were digested with the restriction endonuclease *Sst*I and analyzed by Southern blotting. An ~2 kbp band, which hybridized to an oligonucleotide corresponding to −499 to −481 of the promoter,[6] was isolated, subcloned, and DNA sequenced in both directions. It contained a total of 1218 bp upstream from the start of transcription. The sequence is in agreement with the recently published sequence[7] except for a G/C switch at position −1204 and −1203. Computer analysis (Program Manual for the Wisconsin Package, Version 8, Genetics

FIG. 1. Sequence of human biglycan 5′-flanking DNA. Putative binding sites for *trans*-acting factors are underlined.

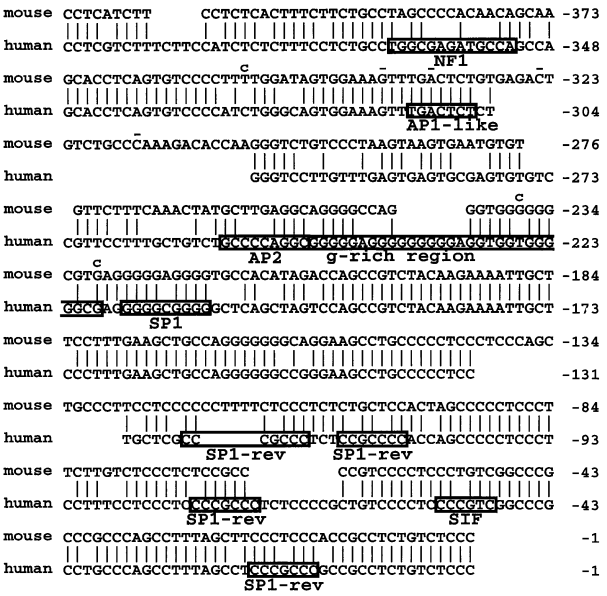

FIG. 2. Comparison of the 5′-flanking regions of the mouse and human biglycan genes. Identical nucleotides are connected with lines; putative binding sites in the human promoter region are boxed. The dashes (-) above the mouse sequence indicate bases not reported in the sequence recently published,[34] and the c's above the mouse sequence show bases that differ.

Computer Group, Madison, WI, U.S.A.) of the biglycan 5′ DNA revealed two clusters of putative binding sites for *trans*-acting factors (Fig. 1) With one region extending 428 bp upstream from the start of transcription and containing a NF1-like site,[26] an AP1-like site,[27] an AP-2 site,[28] a SIF element, and several putative SP1 sites.[29] The other region is situated between –718 and –620 and contains the consensus sequence for the binding sites for C/EBP,[30] AP1 (two sites), and SIF.

The putative mouse promoter region was cloned from a mouse genomic library using the rat biglycan cDNA as probe.[16] Five clones were isolated and analyzed. A 1 kbp *Bam*HI–*Sac*I fragment contained part of intron 1, exon 1, and ~550 bp 5′ sequence. Comparisons were made to the mouse biglycan cDNA sequence[31] and the human gene.[6] With few exceptions, the 5′ sequence was identical to the sequence recently published[32] (Fig. 2) Computer analysis[27] revealed that within the first 400 bases upstream from the transcriptional start site of the human gene the two biglycan promoter regions are 81% homologous (Fig. 2)

Transfection of normal human fibroblasts and fibroblasts with sex chromosome anomalies

To investigate whether the human promoter could activate transcription and to identify possible regulatory elements, 10 progressive deletional promoter-CAT-constructs were made (Fig. 3) PCR was used to create DNA fragments with different 5′ ends to include or exclude promoter elements. The 3′ ends included exon 1 and 91 bp of intron 1, thereby contributing a splice donor to the construct, with the CAT-vector providing the corresponding splice acceptor. The RNA could thus be spliced from the biglycan splice donor to the SV40 splice acceptor. Because such a chimeric construct has, in other genes, been shown to enhance CAT expression,[17] the CAT expression of the –428 construct was compared with the expression of a similar construct in which the splice donor was omitted. However, no difference in CAT expression was found (data not shown).

All the 10 different deletional promoter constructs were able to promote transcription in normal human dermal fibroblasts; this was measured as an increase in CAT activity compared with a CAT construct without promoter (Fig. 3). The increase varied from 9- to 43-fold depending on the promoter fragment. The shortest fragment tested contained 218 bp upstream from the start of transcription and the longest construct contained 1208 bp of biglycan 5′ DNA. The –1208 construct was significantly less active than most of the shorter constructs, suggesting the presence of upstream repressor activity. The approximate locations of at least two other regulatory elements possibly involved in repressor activity were also identified. These elements are located in the areas –497 to –428 and –801 to –713. Significant increases in activity with the constructs –218, –262, –428, –675, and –873 indicate the presence of multiple regulatory elements that are targets for activators. Some of

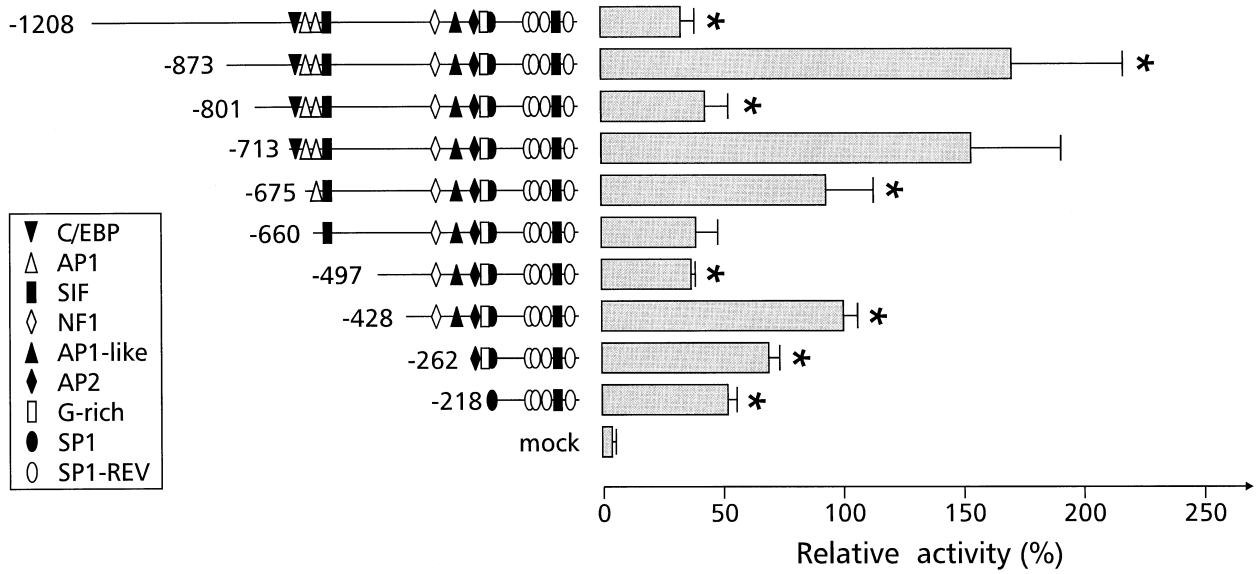

FIG. 3. Human biglycan promoter activity. The left part of the figure illustrates the progressive deletional human biglycan 5′-flanking DNA-CAT constructs used for transient transfections in human dermal fibroblasts. The location of putative binding sites for *trans*-acting factors is indicated by symbols. The right part of the figure shows the promoter activity for the different constructs. CAT activity was divided by luciferase activity and expressed in percentage relative to the activity of construct –428, which was arbitrarily set at 100%. The error bars represent the standard error of the mean, and the stars indicate a statistically significant difference in activity for a promoter construct compared with the construct directly below it. Statistical analysis was performed using analysis of variance and pairwise comparison by Tukey. Experiments were done in triplicate and repeated between three to six times.

these could be AP1, AP2, NF1, SIF, and C/EBP's since the addition of 5′ biglycan DNA containing these sites coincides with an increase in biglycan promoter activity.

RNAse protection assay of the biglycan-CAT transcripts was performed to establish that the differences in CAT activity among the different biglycan promoter-CAT-constructs did not arise from use of different initiation sites and that the initiation sites used were the same as previously determined for the endogenous gene.[6,7] Poly(A) RNA was isolated from MG-63 cells which had been transfected with either the –262 biglycan-promoter-CAT construct or the –1206 biglycan-promoter-CAT construct. The constructs display the same promoter activity whether they are transfected into fibroblasts or MG-63 cells (data not shown). The RNA was hybridized to an antisense probe which included 185 bp upstream from the transcriptional start site of the biglycan gene as well as 67 bp of the CAT gene. The result of the RNAse protection assay (Fig. 4) showed that the transcripts from the two different constructs are initiated at the same major site and that the pattern of protected fragments with one predominant band surrounded by minor bands is the same as seen with the endogenous gene.[6,7] In addition, the more intense signal that was generated with the protected –262 construct transcripts as compared with the –1208 construct transcripts corresponded to the results obtained from the transfection experiments where the –262 construct showed a 2-fold higher activity than the –1208 construct (Fig. 3).

Individuals with abnormal numbers of sex chromosomes have altered biglycan mRNA and protein expression compared with normal controls. The level of biglycan expression correlates to the number of sex chromosomes such that Turner females (45,X) have the lowest levels of biglycan and patients with supernumerary sex chromosome the highest.[9] We therefore wanted to examine the transcriptional regulation of biglycan in cells from individuals with sex chromosome anomalies. Transfections were performed with dermal fibroblasts from either Ullrich-Turner females (45,X, age 8 years), individuals with supernumerary sex chromosomes (47,XXX, age 2 months; 49,XXXXY, age 9 years) or normal fibroblasts (46,XX, ages 2 month and 24 years). Four different biglycan promoter constructs were used. There was an increased activity of the –262 biglycan promoter construct in fibroblasts derived from patients with the genotype 47,XXX (Fig. 5) and 49,XXXXY (data not shown) compared to either normal fibroblasts or fibroblasts from Turner females. This observation indicates that important regulatory activity may be contained in the 44 bp sequence that resides between –262 and –218 in the human biglycan promoter. For this reason, we decided to focus on the 44 bp –262 to –218 region in our further studies of the transcriptional regulation of human biglycan.

DNAse footprinting and electrophoretic mobility shift assays

The binding sites located in and around the 44 bp –262 to –218 region in the human biglycan 5′ DNA were analyzed using DNAse footprinting and EMSA. The 44 bp region contains an AP2 consensus sequence and a G-rich sequence

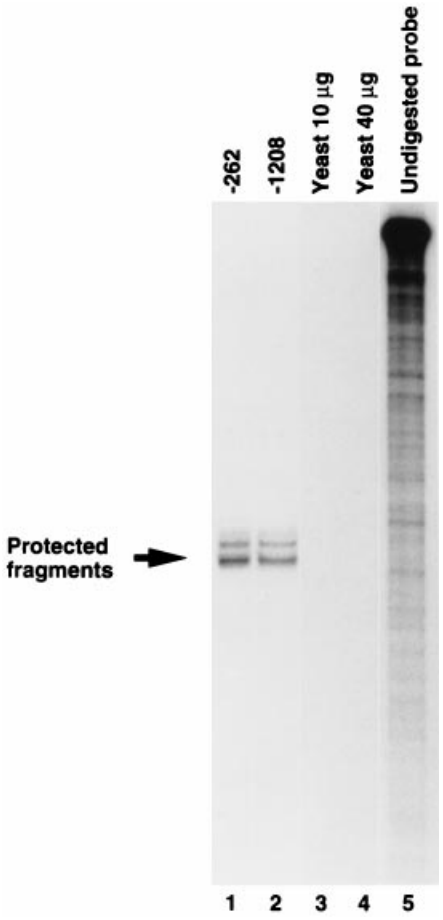

FIG. 4. RNAse protection assay to determine the initiation sites of the transcripts derived from the –262 biglycan-promoter-CAT-construct and the –1208 biglycan-promoter-CAT-construct. The labeled antisense probe was hybridized to various RNA and then digested with the RNAse A/RNAse T1 solution (1:100 dilution) as described in Materials and Methods. The sources and amounts of RNA used were as follow: 1.3 μg poly(A) RNA from cells transfected with the –262 promoter-construct (lane 1), 1.6 μg poly(A) RNA from cells transfected with the –1208 promoter-construct (lane 2), 10 μg of torulla yeast RNA (lane 3), 40 μg of torulla yeast RNA (lane 4). In lane 5 is the undigested probe; this is not in scale with the rest of the figure.

with resemblance to the c-Krox binding site (Fig. 7B). Just downstream for the G-rich site is an Sp1 consensus site. DNAse footprinting with nuclear extracts showed a strong footprint corresponding to the G-rich site and extending partially onto the AP2 site and another footprint corresponding to the Sp1 site just downstream (Fig. 6) DNAse footprinting with recombinant Sp1 confirmed the binding of Sp1 to the site at -216 to -208 and additionally showed a weak protection of the G-rich site (Fig. 6, lanes 8 and 9). Although the AP2 site was only partially protected in the footprinting with nuclear extract, recombinant human AP2 binds specifically to the site (Fig. 6, lanes 5, 6, and 7). Electrophoretic mobility shift assays confirmed the binding pattern of the DNAse footprinting and contributed additional information about the G-rich site. A 44 bp (–262 to –218) biglycan DNA oligonucleotide was used as probe and incubated with nuclear extracts from either MG-63 cells, human dermal fibroblasts, HeLa cells, or WI-38 cells (Fig. 7A), all with similar binding patterns (data not shown). Three DNA–protein complexes were seen, two slow migrating (I and II, Fig. 7A) and one faster migrating complex (III, Fig. 7A). All three complexes represent specific binding activity. In accordance with the DNAse footprinting, AP2 is probably not part of the complexes because the wild-type 44 bp oligonucleotide and an oligonucleotide with a mutated AP2 site competed equally well in the binding experiments and an oligonucleotide with an AP2 consensus sequence did not compete for binding.

Oligonucleotides that had either a mutation in the central part of the G-rich site (Δ44-G) or to the 3′ end of the site (Δ44-GT) were then used as competitors in the EMSA. The Δ44-GT oligonucleotide competed as well as the wild-type and the Δ44-AP2 oligonucleotides, only the Δ44-G oligonucleotide could not inhibit binding to the wild-type probe. An Sp1 consensus oligonucleotide also did not work as a competitor. Taken together, these results suggest that the G-rich site and not the AP2 site in the biglycan promoter is a binding site for one or more nuclear factors. The G-rich site is similar to the c-Krox binding site in the mouse α1 (I) collagen promoter (Fig. 7B), and in the EMSA the α1 (I)coll oligonucleotide was equally efficient as competitor for the binding activity of the fast migrating complex (Fig. 7A, III, lanes 11 and 12) as the 44 bp oligo. This indicated that the G-rich site is a c-Krox binding site, and to test this, EMSA was performed with cell extracts from COS cells that had been transfected with a sense or antisense human c-Krox expression vector. When the 44 bp biglycan probe was used, only extracts from COS cells transfected with the sense c-Krox expression vector resulted in a mobility shift Fig. 8A). The binding could be competed with the addition of either the unlabeled 44 bp probe or the α1 (I)coll oligonucleotide, but not with an Sp1 or AP1 consensus oligonucleotide. The extracts from the sense or antisense transfected COS cells were of equally good quality as shown by EMSA. (Fig. 8B). In this experiment, incubation of the c-Krox antisense or sense extracts with a probe that contained the Sp1 consensus sequence resulted in an identical binding pattern. The upper two bands represent specific binding as shown with the addition of a 100-fold excess unlabeled Sp1 oligonucleotide (Fig. 8B, arrows). These results further support the hypothesis that c-Krox binds to the human biglycan promoter.

Detection of c-Krox mRNA in human osteoblastic cells

The experiments described above indicated that c-Krox might be involved in the transcriptional regulation of biglycan and, since c-Krox has only been shown to be expressed in fibroblasts, we wanted to investigate whether other biglycan-expressing cells express c-Krox. mRNA from MG-63 cells, human osteoblastic cells, and human bone marrow stromal cells was reverse transcribed and PCR amplified (RT-PCR) with human c-Krox–specific primers. The products were analyzed by agarose gel electrophoresis. A single

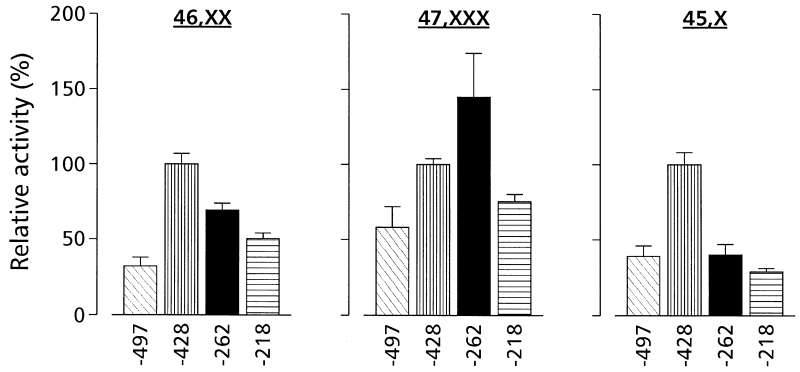

FIG. 5. The promoter activity of four different human biglycan promoter constructs when transiently transfected into human dermal fibroblasts with the genotype 46,XX, 47,XXX, or 45,X. CAT activity was divided by luciferase activity and expressed in percentage relative to the activity of construct –428 which was arbitrarily set at 100%. The error bars represent the standard error of mean. Experiments were done in triplicate and repeated three times.

band of the expected size of 308 bp[24] was obtained from all three cell types (Fig. 9A). No product was obtained when the reverse transcriptase was omitted from the reaction. Subcloning and DNA sequencing of the 308 bp product confirmed that it was human c-Krox.[24] To further verify these results, a Northern blot was made with total RNA isolated from either MG-63 cells, WI-38 cells, or human osteoblastic cells. Hybridization with a radiolabeled human c-Krox cDNA[24] detected a ~3.4 kb transcript in all three cell types (Fig. 9B), and this size corresponds to the ~3.5 kb transcript detected in mouse fibroblasts.[13]

DISCUSSION

Although the function of biglycan is currently unknown, evidence is emerging which points toward a role in the organization of the extracellular matrix,[4] a function as a growth factor binding protein,[3] and a possible participant in osteoblast differentiation.[1] The mechanisms involved in the transcriptional regulation of the human biglycan gene have just recently begun to be elucidated.[6,7] In the present study, human dermal fibroblasts were transfected with progressive deletional biglycan 5′-flanking DNA constructs, and a significant variation in activity among the individual constructs were found. A small deletion often caused a more than 2-fold increase or decrease in promoter activity, indicating the presence of repressor or activator activity. The deleted DNA sequences implicated in repressor activity, in all cases, do not contain consensus sequences for any known *trans*-acting factors, indicating the presence of novel *cis*-acting elements. On the contrary, the DNA that gave rise to significant increases in promoter activity contains the putative binding sites for many well characterized activators. A total of 1208 bp upstream from the transcriptional start site were analyzed, and the –1208 construct consistently had very low activity, indicating that more upstream (or downstream) DNA may be required for the complete transcriptional regulation of the biglycan gene. In addition, the current study does not elucidate which elements that might be responsible for cell-specific expression of human biglycan. The computer comparison of the 5′-flanking DNA of mouse and human biglycan showed that they are highly homologous (Fig. 2) however, even though most of the Sp1 sites are well conserved, the more upstream elements are

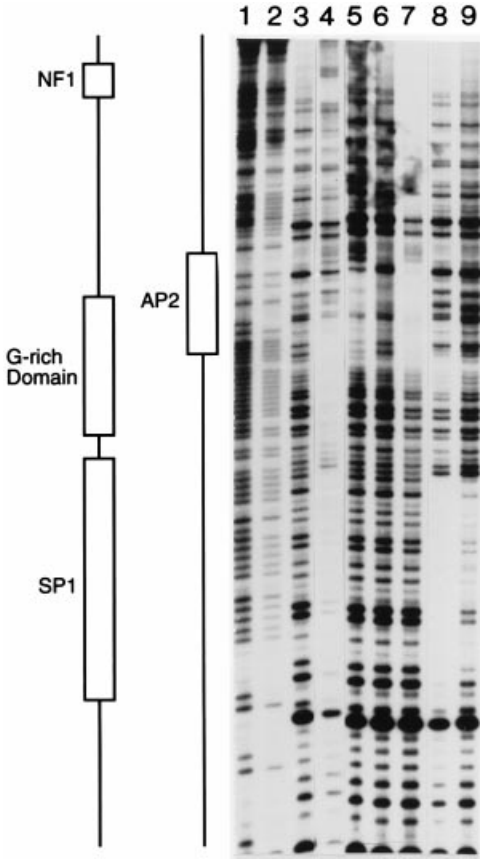

FIG. 6. DNAse footprinting analysis of the human biglycan 5′-flanking DNA from –428 to –188. The diagram to the left depicts the position of the different putative binding sites as concluded from the Maxam–Gilbert sequencing reaction; the C/T and C reactions are shown in lanes 1 and 2, respectively. For the binding experiments, the probe was incubated as follows: buffer alone (lane 3), 40 μg of HeLa extract (from Promega, lane 4), ~50 ng of AP-2 protein (from Promega, lane 5), ~50 ng of AP-2 protein with 100-fold molar excess 44 bp –262 to –218 biglycan oligonucleotide (lane 6), ~50 ng of AP-2 protein with 100- fold molar excess 44 bp –262 to –218 biglycan oligonucleotide with a mutated AP2 site (Δ44-AP2, lane 7), ~50 ng of SP1 protein (from Promega, lane 8), ~50 ng of SP1 protein with 100-fold molar excess of a SP1 consensus oligonucleotide (from Promega, lane 9).

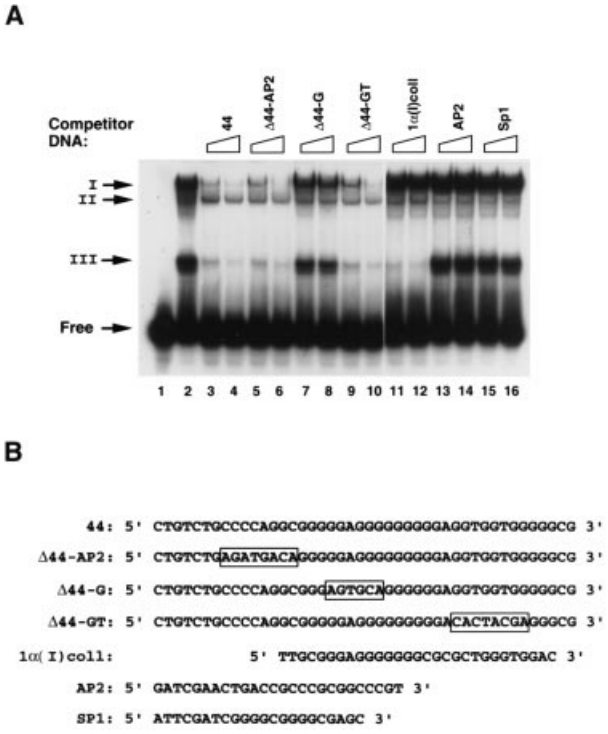

FIG. 7. (A) Electrophoretic mobility shift assay. The biglycan –262 to –218 (44) oligonucleotide was used as probe and incubated with nuclear extracts from WI-38 fibroblasts (lanes 2–16). Lane 1 shows the migration of the probe without addition of protein. Fifty- and 100-fold molar excess of competitor oligonucleotides were used (lanes 3–16). (B) The sequences of the different double-stranded DNA oligonucleotides used in the binding experiments. The 44 is the –262 to –218 biglycan sequence; various mutations (boxed) have been introduced in the following three oligonucleotides. 1α(I)coll contains the sequence of the c-Krox binding site in the mouse α1 (I)collagen promoter. This binding site was used for the cloning of mouse c-Krox.[13]

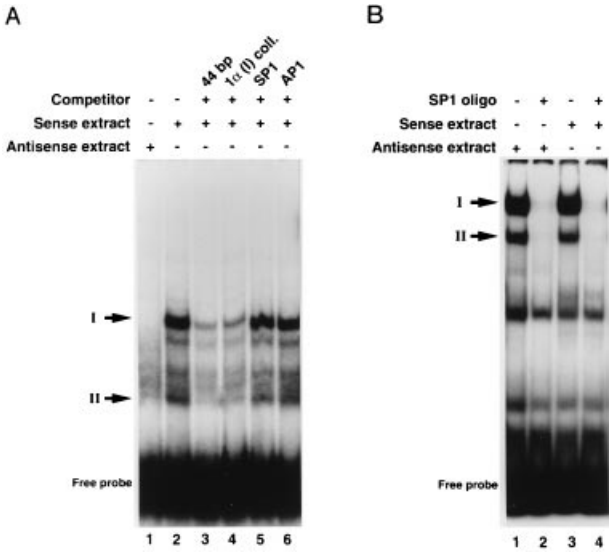

FIG. 8. Electrophoretic mobility shift assays. (A) The biglycan 44 bp probe was incubated with 5 μg of whole cell extract from COS cells transfected with either an antisense (lane 1) or sense c-Krox expression vector (lanes 2–6). Various oligonucleotides were used as competitors in 100-fold molar excess (lanes 3–6). (B) A labeled Sp1 consensus sequence oligonucleotide was incubated with 5 μg of whole cell extract from the c-Krox antisense (lanes 1 and 2) or sense (lanes 3 and 4) expressing COS cells. A 100-fold molar excess of the Sp1 oligonucleotide was used as competitor (lanes 2 and 4).

not. An example is the NF1 site in the human gene, which is protected in the DNAse footprinting (Fig. 6) but which does not seem to be present at the same position in the mouse gene.

To elucidate the transcriptional regulation of the biglycan gene in individuals with sex chromosome anomalies, fibroblasts from either 45,X, 46,XX, 47,XXX, or 49,XXXXY donors were transiently transfected with biglycan 5′-flanking DNA connected to a reporter gene. A differential regulation of the biglycan promoter was found implicating the –262 to –218 region of the biglycan 5′-flanking gene in the regulation. These experiments revealed a potentially interesting regulatory area of the biglycan gene, and for this reason the –262 to –218 region was analyzed in detail. DNAse footprinting and electrophoretic mobility assays identified the G-rich site located at approximately –248 to –230 to be the major nuclear factor binding site. No binding was found to the AP2 site, and transient transfections of human dermal fibroblasts with constructs with a mutated AP2 site showed no change in transcriptional activity compared with the wild-type construct (data not shown). Recently the transcription factor c-Krox (collagen Krox protein) was discovered[13]; it binds to sites in the type I collagen promoters and regulates transcription.[14] Because its binding site is very similar to the biglycan G-rich site (Fig. 7B), we decided to examine whether c-Krox could bind to this sequence in the biglycan promoter. We subsequently showed that at least one of the factors that binds the biglycan G-rich site also binds the α1 (I)collagen c-Krox site and that recombinant human c-Krox can bind specifically to the biglycan site. Taken together, we suggest that the biglycan G-rich site is a c-Krox binding site.

The observation that EMSA of nuclear extracts from human dermal fibroblasts and human primary osteoblastic cells were similar suggested that c-Krox might also be expressed in osteoblasts. The human c-Krox transcript was then identified in primary osteoblastic cells, in MG-63 cells, and human bone marrow stromal cells, both by RT-PCR and Northern blotting, suggesting that these cell types express c-Krox and that c-Krox like Krox-20[33] might play a role in bone. The few studies that have been performed to examine c-Krox expression have been done exclusively in mice,[13,14] where the c-Krox mRNA was found predominantly in skin and to a lesser degree in heart, smooth muscle, liver, and spleen; no expression was detected in bone. In our hands, the c-Krox expression was equal if not more pronounced in osteoblasts compared with fibroblasts, and it is possible this might reflect a different expression in humans compared with mice.

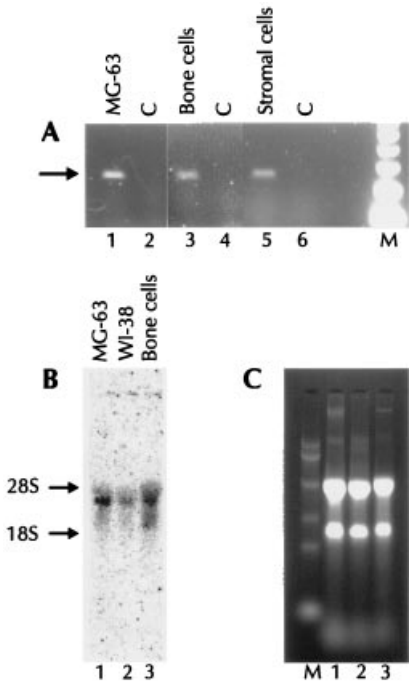

FIG. 9. (A) RT-PCR with c-Krox–specific primers, in the control reactions (lane 2, 4, and 6) the reverse transcriptase was omitted from the cDNA synthesis. MG-63 is an osteosarcoma cell line; bone and stromal cells are human primary osteoblastic cells and human bone marrow stromal cells, respectively. The arrow shows the location of the 308 bp PCR product. "M" indicates the lane with the 123 bp ladder; the size of the bands increases with 123 bp for each step, beginning with 123 bp at the bottom of the gel. (B) Northern blot analysis of human c-Krox transcript, 20 μg of total RNA was loaded in each lane. The arrow indicates the position of the c-Krox mRNA at ~3.4 kb, just below 28S rRNA. (C) The RNA gel was used for Northern blot analysis. Lanes 1, 2, and 3 correspond to lanes 1, 2, and 3 in Fig. 8B. "M" indicates the lane with the RNA markers with the sizes 0.24, 1.35, 2.37, 4.40, 7.46, and 9.49 kb (GIBCO BRL Life Technologies).

It would be interesting to determine whether c-Krox plays a role in the phenotype of Turner syndrome. To date, the chromosomal localization of the human gene is not known, but mouse c-Krox has been mapped to the (A1–A2) region of the X chromosome.[14] An argument against involvement of c-Krox in the Ullrich-Turner syndrome is the observation that collagen mRNA expression does not seem to be affected in dermal fibroblasts from females with the syndrome.[9] However, the lack of c-Krox expression in mouse bone does not exclude c-Krox as a candidate since mice that are X0, in contrast to humans, are viable, fertile, and anatomically normal.[34] Another pathological condition where c-Krox might play a role is fibrosis, for example in keloids there is a coordinate induction of type I collagen and biglycan expression, but normal expression of decorin.[35] Decorin is a small proteoglycan that is 55% homologous to biglycan[15] and that often is present in the same tissues as biglycan.[1] The homology of the two genes does not extend to the 5′ flanking DNA,[36] and using computer searches (Program Manual for the Wisconsin Package, Version 8, Genetics Computer Group), we have not found the sequence of a c-Krox binding site in the decorin gene.

In summary, our data provide evidence for a diverse transcriptional regulation of the human biglycan gene which possibly is affected in individuals with sex chromosome anomalies. In addition, we have shown that the transcription factor c-Krox has a binding site in the biglycan promoter and that c-Krox is expressed in human primary osteoblastic cells. This study provides a basis for further studies of the expression and role of c-Krox in human bone.

ACKNOWLEDGMENTS

This work was supported in part by the Danish Medical Research Council (project no. 12-9572-2). We thank Dr. C.C. Baker for the CAT expression vectors and for good discussions, Dr. J.M. Liu for the luciferase expression vector, Dr. K.L. Dreher for the rat biglycan cDNA, Dr. T.C. Doetschman for the mouse cDNA library, and Drs. G. Karsenty, N.T. Foged, K. Jensen, and T. Livelli for helpful comments and discussions. We thank Ms. A.W. Vestergaard for excellent technical assistance. The nucleotide sequence data reported in this paper have been submitted to GenBank under the accession numbers U82940 and U82941.

REFERENCES

1. Bianco P, Fisher LW, Young MF, Termine DJ, Gehron Robey P 1990 Expression and localization of the two small proteoglycans biglycan and decorin in developing human skeletal and non-skeletal tissues. J Histochem Cytochem **38:**1549–1563.
2. Young MF, Ibaraki K, Kerr JM, Heegaard A-M 1993 Molecular and cellular biology of the major noncollagenous proteins in bone. In: Noda M (ed.) Cellular and Molecular Biology of Bone. Academic Press, Inc., San Diego, CA, U.S.A., pp. 191–234.
3. Hildebrand A, Romaris M, Rasmussen LM, Heinegård D, Twardzik DR, Border WA, Rouslahti E 1994 Interaction of the small interstitial proteoglycans biglycan, decorin and fibromodulin with transforming growth factor β. Biochem J **302:**527–534.
4. Schönherr E, Witsch-Prehm P, Harrach B, Robenek H, Rauterberg J, Kresse H 1995 Interaction of biglycan with type I collagen. J Biol Chem **270:**2776–2783.
5. Yamaguchi Y, Mann DM, Rouslahti E 1990 Negative Regulation of transforming growth factor-β by the proteoglycan decorin. Nature **346:**281–284.
6. Fisher LW, Heegaard A-M, Vetter U, Vogel W, Just W, Termine JD, Young MF 1991 Human biglycan gene putative promoter, intron-exon junctions and chromosomal localization. J Biol Chem. **266:**14371–14377.
7. Ungefroren H, Krull NB 1996 Transcriptional regulation of the biglycan gene. J Biol Chem **271:**15787–15795.
8. Traupe H, van den Ouweland AMW, van Oost BA, Vogel W, Vetter U, Warren ST, Rocchi M, Darlison MG, Ropers H-H 1992 Fine mapping of the human biglycan (BGN) gene within

the Xq28 region employing a hybrid cell panel. Genomics **13:**481–483.
9. Geerkens C, Vetter U, Just W, Fedarko NS, Fisher LW, Young MF, Termine JD, Gehron Robey P, Wöhrle D, Vogel W 1995 The X-chromosome human biglycan gene BGN is subject to X inactivation but is transcribed like a X-Y homologous gene. Hum Genet **96:**44–52.
10. Rao E, Weiss B, Mertz A, Meder J, Schiebel K, Ogata T, Matsuo N, Rappold GA 1995 Construction of a cosmid contig spanning the short stature candidate region in the pseudoautosomal region PAR 1. In: Albertsson-Wikland K, Ranke M (eds.) Turner Syndrome in a Life-Span Perspective. Elsevier Science B.V., pp. 19–24.
11. Turner HH 1938 A syndrome of infantilism, congenital webbed neck, and cubitus valgus. Endocrinology **23:**566-574.
12. Hall JG, Lopez-Rangel E 1995 In: Albertsson-Wikland K, Ranke M (eds.) Turner Syndrome in a Life-Span Perspective. Elsevier Science B.V., pp. 3–9.
13. Galéra P, Musso M, Ducy P, Karsenty G 1994 c-Krox, a transcriptional regulator of type I collagen gene expression, is preferentially expressed in skin. Proc Natl Acad Sci USA **91:**9372–9376.
14. Galéra P, Park R-W, Ducy P, Mattéi M-G, Karsenty G 1996 c-Krox binds to several sites in the promoter of both mouse type I collagen genes. J Biol Chem **271:**21331–21339.
15. Fisher LW, Termine JD, Young MF 1989 Deduced protein sequence of bone small proteoglycan I (biglycan) shows homology with proteoglycan II (decorin) and several nonconnective tissue proteins in a variety of species. J Biol Chem 264:4571–4576.
16. Dreher KL, Asundi V, Matzura D, Cowan K 1990 Vascular smooth muscle biglycan represents a highly conserved proteoglycan within the arterial wall. Eur J Cell Biol **53:**296–304.
17. Baker CC 1990 An improved chloramphenicol acetyltransferase expression vector system for mapping transcriptional and post-translational regulatory elements in animal cells. In: Alitalo KK, Huhtala ML, Knowles J, Vaheri A (eds.) Recombinant Systems in Protein Expression. Elsevier Science Publishers B.V., pp. 75–86.
18. Gehron Robey P, Termine JD 1985 Human bone cells *in vitro*. Calcif Tissue Int **37:**453–460.
19. Kuznetsov S, Gehron Robey P 1996 Species differences in growth requirements for bone marrow stromal fibroblast colony formation *in vitro*. Calcif Tissue Int **56:**265–270.
20. Kingston RE, Chen CA, Okayama H 1992 Transfection of DNA into eukaryotic cells. In: Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (eds.) 1991 Current Protocols in Molecular Biology. John Wiley & Sons, Inc., New York, NY, U.S.A., pp. 9.1.1–9.1.9.
21. Liu JM, Fujii H, Green SW, Komatsu N, Young NS, Shimada T 1991 Indiscrimate activity from the B19 promoter in nonpermissive cells. Virology **182:**361–364.
22. Gilman M 1991 Removal of contaminating DNA. In: Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (eds.) 1991 Current Protocols in Molecular Biology, John Wiley & Sons, Inc., New York, NY, U.S.A., pp. 4.1.4–4.1.5.
23. Abmayr SM, Workman JL 1993 Preparation of nuclear and cytoplasmic extracts from mammalian cells. In: Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (eds.) 1991 Current Protocols in Molecular Biology, John Wiley & Sons, Inc., New York, NY, U.S.A., pp. 12.1.1–12.1.9.
24. Widom RL, Culic I, Lee J, Korn KH 1997 Cloning and characterization of hcKrox, a transcriptional regulator of extracellular matrix gene expression. Gene (in press).
25. Ibaraki K, Termine JD, Whitson WS, Young MF 1992 Bone matrix mRNA expression in differentiating fetal bovine osteoblasts. J Bone Miner Res **7:**743–754.
26. Gronostajski RM 1987 Site-specific DNA binding of nuclear factor I: Effect of spacer region. Nucleic Acids Res **15:** 5545–5559.
27. Lee W, Mitchell P, Tjian R 1987 Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. Cell **49:**741–752.
28. Mitchell PJ, Wang C, Tjian R 1987 Positive and negative regulation of transcription in vitro: Enhancer-binding protein AP-2 is inhibited by SV40 T antigen. Cell **50:**847–861.
29. Kadonaga JT, Carner KR, Masiarz Tjian R 1987 Isolation of cDNA encoding transcription factor Sp1 and functional analysis of the DNA binding domain. Cell **51:**1079–1090.
30. Johnson PF, Landschulz WH, Graves BJ, McKnight SL 1987 Identification of a rat liver nuclear protein that binds to the enhancer core element of three animal viruses. Genes Dev **1:**133–146.
31. Rau W, Just W, Vetter U, Vogel W 1994 A dinucleotide repeat in the mouse biglycan gene (EST) on the X chromosome. Mamm Genome **5:**395–396.
32. Wegrowski Y, Pillarisetti J, Danielson KG, Suzuki S, Iozzo RV 1995 The murine biglycan: complete cDNA cloning, genomic organization, promoter function, and expression. Genomics **30:**8–17.
33. Levi G, Topilko P, Schneider-Maunoury S, Lasagna M, Mantero S, Cancedda R, Charnay P 1996 Defective bone formation in *Krox*-20 mutant mice. Development **22:**113–120.
34. Ashworth A, Rastan S, Lovell-Badge R, Kay G 1991 X-chromosome inactivation may explain the difference in viability of X0 humans and mice. Nature **351:**406–408.
35. Hunzelmann N, Anders S, Sollberg S, Schönherr E, Krieg T 1996 Co-ordinate induction of collagen type I and biglycan expression in keloids. Br J Dermatol **135:**394–399.
36. Santra M, Danielson KG, Iozzo RV 1994 Structural and functional characterization of the human decorin gene promoter. J Biol Chem **269:**579–587.

Address reprint requests to:
Anne-Marie Heegaard, M.D.
Center for Clinical and Basic Research
Ballerup Byvej 222
DK-2750 Ballerup, Denmark

Received in original form December 20, 1996; in revised form June 30, 1997; accepted August 1, 1997.